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Uncovering tropical diversity: six sympatric cryptic species of *Blepharoneura* (Diptera: Tephritidae) in flowers of *Gurania spinulosa* (Cucurbitaceae) in eastern Ecuador

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Diversification of phytophagous insects is often associated with changes in the use of host taxa and host parts. We focus on a group of newly discovered Neotropical tephritids in the genus *Blepharoneura*, and report the discovery of an extraordinary number of sympatric, morphologically cryptic species, all feeding as larvae on calyces of flowers of a single functionally dioecious and highly sexually dimorphic host species (*Gurania spinulosa*) in eastern Ecuador. Molecular analyses of the mitochondrial cytochrome oxidase-I gene from flies reared from flowers of *G. spinulosa* reveal six distinct haplotype groups that differ by 7.2–10.1% bp (uncorrected pairwise distances; $N = 624$ bp). Haplotype groups correspond to six distinct and well-supported clades. Members of five clades specialize on the calyces of flowers of a particular sex: three clades comprise male flower specialists; two clades comprise female flower specialists; the sixth clade comprises generalists reared from male and female flowers. The six clades occupy significantly different morphological spaces defined by wing pigmentation patterns; however, diagnostic morphological characters were not discovered. Behavioural observations suggest specific courtship behaviours may play a role in maintaining reproductive isolation among sympatric species. Journal compilation © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **93**, 779–797. No claim to original US government works.

ADDITIONAL KEYWORDS: courtship – dioecy – host specificity – host use – mtDNA – Neotropics – phytophagous insects – reproductive isolation – speciation – wing pattern.

INTRODUCTION

In the past decade, great theoretical advances have been made in understanding how biodiversity is generated and maintained (Rosenzweig, 1995; Avise, 2000; Hubbell, 2001; Brooks & McLennan, 2002; Webb *et al*., 2002; Thompson, 2005). Yet most of the raw material (i.e. the biodiversity itself) remains undiscovered and undescribed: conservative estimates of insect species diversity range between 2 and 8.5 million (Stork, 1988; Hodkinson & Casson, 1991; Basset *et al*., 1996; Novotny *et al*., 2002; Grimaldi & Engel, 2005) – which is greater than the total number of described species on Earth (Wilson, 1992). These estimates are conservative, in part because they do not incorporate data on numbers of cryptic species (i.e. species that are very similar or indistinguishable

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morphologically). Although cryptic species are often discovered in economically important groups of insects (e.g. Perring *et al*., 1993; Munstermann & Conn, 1997; Scheffer, 2000; Scheffer & Lewis, 2001; Brunner *et al*., 2004), cryptic species are also discovered in groups in which: (1) sexual selection leads to the rapid evolution of behaviours, but not of morphology (Henry, 1994; Wells & Henry, 1998; Mendelson & Shaw, 2005); (2) host specificity is associated with diversification (Bush, 1966; Condon & Steck, 1997; Berlocher, 2000; Scheffer & Wiegmann, 2000; Favret & Voegtlin, 2004; Hebert *et al*., 2004); and (3) both host specificity and mating behaviours contribute to diversification (Wood, 1980; Wood & Keese, 1990; Rodriguez, Sullivan, & Cocroft, 2004). Although proportionately more cryptic species complexes have been discovered in the temperate zone than in the tropics (Bickford *et al*., 2006), most authors agree that the majority of tropical insects remain undescribed (Gaston, 1991). Because most assessments of undescribed diversity are based on the rate of discovery of morphologically distinct species, discovery of morphologically cryptic species may dramatically increase estimates of diversity (Condon, 1994; Hebert *et al*., 2004; Bickford *et al*., 2006; Smith *et al*., 2007).

Nearly half of all described species of insects are phytophagous (Strong, Lawton, & Southwood, 1984). What accounts for the extraordinary diversity of phytophagous insects? Diversification of phytophagous insects is often associated with changes in their use of host taxa (Ehrlich & Raven, 1964; Bush, 1969; Mitter, Farrell, & Futuyma, 1991; Futuyma & Keese, 1992; Scheffer & Wiegmann, 2000; Stireman, Nason, & Heard, 2005; Novotny *et al*., 2006). Clearly, affiliations with particular plant taxa are important; however, change in use of host parts may also play an important role in diversification in some insect groups (e.g. Cecidiomyiidae, Gagné & Waring, 1990; sawflies, Nyman, Widmer, & Roininen, 2000; gall wasps, Ronquist & Liljeblad, 2001; Cook *et al*., 2002; beetles, Marvaldi *et al*., 2002; Farrell & Sequeira, 2004; Morse & Farrell, 2005), especially if the host parts are spatially and temporally isolated, and if courtship and mating takes place on different parts of the plants (Condon & Steck, 1997). Although endophagy – often involving specialization on particular plant parts – appears to slow the rates of diversification in some groups (Nyman *et al*., 2006), factors other than larval host use (e.g. sexual selection) may accelerate diversification in other groups of endophagous phytophagous insects. To explore these possibilities, we focus our attention on *Blepharoneura*, a group of Neotropical tephritids that includes specialists on different parts of plants in the family Cucurbitaceae, and that also includes many morphologically cryptic species with elaborate courtship displays (Condon & Norrbom, 1999).

All species of *Blepharoneura* with known host records feed as larvae inside parts of plants in the Cucurbitaceae, a family characterized by unisexual flowers and dichogamy (temporal separation of male and female flowers). Many species of *Blepharoneura* are specialists on either male or female parts of plants in the Guraniinae, a subtribe characterized by extreme sexual dimorphism: female flowers are produced on leafless pendulous branches; male inflorescences are borne on actively climbing leafy branches; and male and female inflorescences are usually temporally and spatially isolated (Condon & Gilbert, 1988, 1990). Currently, 22 species are recognized within *Blepharoneura*, and it is estimated that the genus includes at least 200 species (Norrbom & Condon, 1999).

Previous studies of communities of *Blepharoneura* revealed multiple sympatric cryptic species feeding on single species of *Gurania* (Condon & Steck, 1997). In Costa Rica, four morphologically cryptic species of *Blepharoneura* infest flowers of *Gurania costaricensis* Cogn.: two species infest female flowers and two species infest male flowers, and species infesting the same host part have different (but overlapping) altitudinal ranges. In northern Venezuela and Trinidad, three species of *Blepharoneura* feed on *Gurania spinulosa* (Poepp. & Endl.) Cogn. (= *Gurania lobata* L.): one species on female flowers, one species on male flowers, and a third species on seeds (Condon & Norrbom, 1994; Condon & Steck, 1997). Two of these three species court and mate on different parts of the host plant; the site of courtship of the third species is unknown (Condon & Norrbom, 1999). Here we report on the diversity of the community of *Blepharoneura* infesting the same species of plant (*G. spinulosa*) within an 8-km radius of the Jatun Sacha Biological Station in the lowlands of eastern Ecuador (Napo province).

This is one of a series of papers reporting the discovery of multiple cryptic sympatric species of *Blepharoneura* in communities throughout the Neotropics. A focus on sympatric populations allows us to demonstrate most clearly that distinct genetic lineages coexist in space: some sharing the same host parts, and some infesting different parts of the same host species. In our first study, we used allozyme electrophoresis (14 enzymes) to reveal morphologically cryptic species (Condon & Steck, 1997). Here we report on the results of analyses of 624 bp of the mitochondrial gene cytochrome oxidase I (COI), a gene that has been useful in the delineation of species (Scheffer & Wiegmann, 2000; Hebert *et al*., 2004; Monaghan *et al*., 2005). Although conclusions drawn from the analyses of single gene regions can

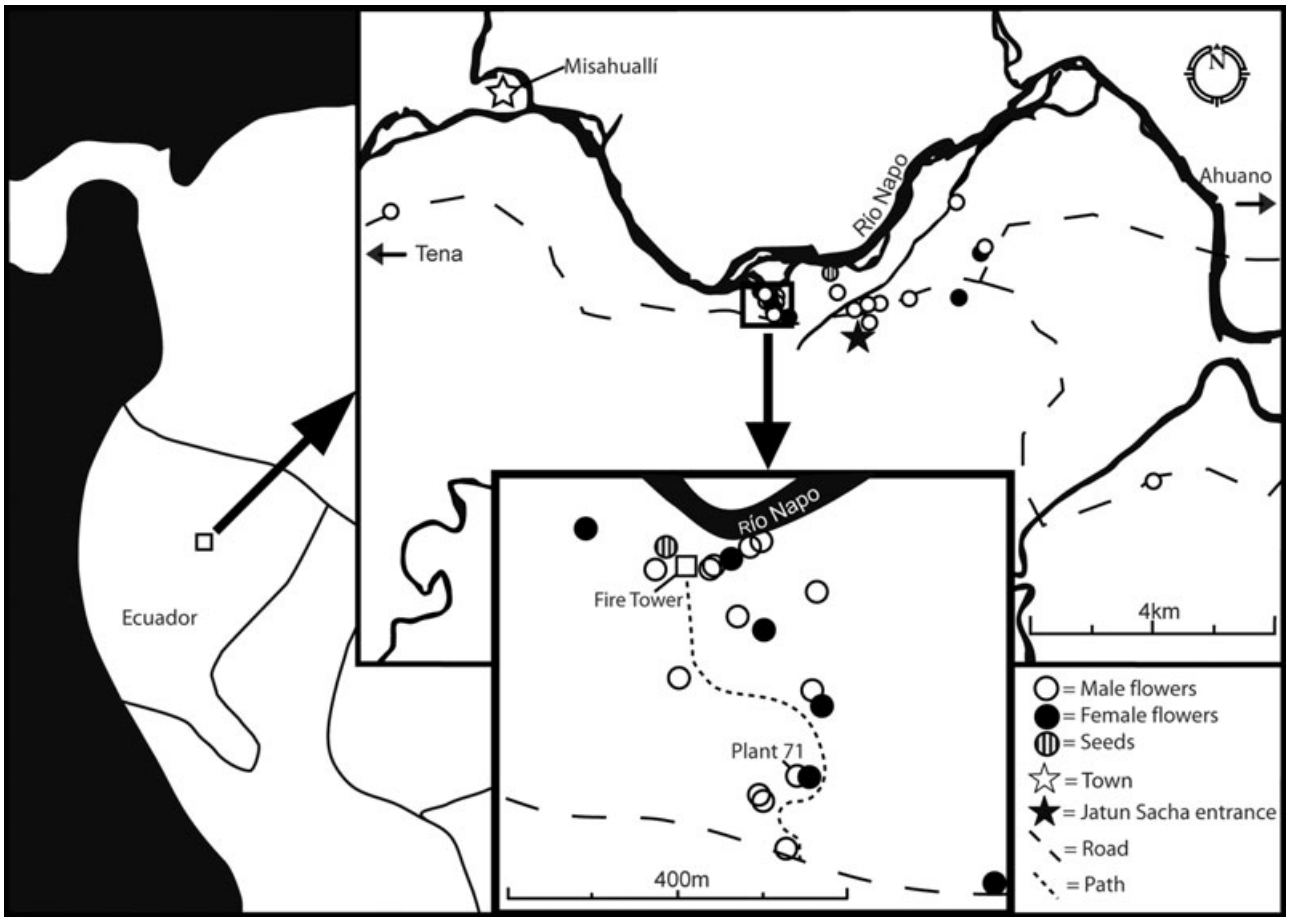

Figure 1. Study site in the Napo Province in eastern Ecuador. The first inset indicates the locations of host plants within 8 km of the Jatun Sacha Biological Station (01°03.941′S, 77°36.998′W) near Misahualli. The second inset indicates the locations of hosts (clumps of *Gurania spinulosa* flowers, including plant #71) within disturbed habitat at the Ishpingo Botanical Garden.

be problematic (Moore, 1995; Doyle, 1997; Hoelzer, 1997), preliminary phylogenetic analyses of two nuclear genes yielded results consistent with those reported here (M. A. Condon, S. J. Scheffer, M. L. Lewis & S. M. Swensen, unpubl. data). Because our current work in diverse tropical communities reveals some geographically widespread lineages, and some apparently endemic lineages, we defer formal descriptions of these lineages as species until we have sampled Neotropical communities more thoroughly, and can better assess the geographical patterns of variation in molecules, morphology, and behaviour.

Our goals in this paper are to: (1) use the mitochondrial gene COI to reveal the diversity of sympatric *Blepharoneura* on *G. spinulosa*; (2) assess patterns of host use; and (3) report preliminary observations on behaviour and morphological characters. We discuss the relevance of these observations to hypotheses about diversification of phytophagous insects.

MATERIAL AND METHODS

Study site

We were based at the Jatun Sacha Biological Station (JSBS), which is located near Misahuallí in the Napo province of eastern Ecuador (Fig. 1). We collected flowers and fruit from plants found along the roadside in disturbed habitat within 8 km of the entrance (01°03.941′S, 77°36.998′W) to JSBS, and also within the JSBS property. The road runs roughly parallel to the river, varies little in elevation (~390–420 m a.s.l), and is bordered by habitat ranging from cattle pastures to forest. Our collecting and observations were most intensive in the disturbed habitat surrounding an area maintained by Jatun Sacha as the Ishpingo Botanical Garden (Fig. 1).

Table 1. Infestation (number of emerged larvae) of cucurbits collected in March 2005

	Male flowers		Female flowers		Fruit	
Cucurbit taxa	#flowers	#larvae	#flowers	#larvae	#fruit	#larvae
Cayaponia sp.	8	0	42	0	62	0
Elateriopsis sp.	117	0	13	0	2	0
Gurania eriantha	444	11	6	1	0	0
Gurania guentheri	30	0	32	0	0	0
Gurania pedata	114	0	60	0	29	0
Gurania rhizantha	127	0	7	0	0	0
Gurania spinulosa	3047	624	657	143	61	10
Sicydium sp.	0	0	0	0	176	0

Collection and rearing

To rear *Blepharoneura*, we collected flowers and fruit of *G. spinulosa* on 6–9 February 1998 and on 5–18 March 2005. In 2005 we broadened our study, and searched the study site for all cucurbit plants bearing flowers and fruits that were within reach. We used 12-m-long collecting poles to collect flowers and fruit in the canopy. If branches climbing in the canopy were inaccessible (> 14-m high), we searched the ground beneath branches for fallen flowers and fruit. We also recorded the locations of each cluster of flowering or fruiting branches (each cluster probably represents a single plant). Because it is not possible to use external cues to determine whether a flower or fruit is infested with *Blepharoneura*, we collected all flowers and fruit that we encountered (Table 1). Individual flowers or fruit were placed in small plastic cups, which were checked daily for larval emergence. When larvae emerged and pupariated, individual puparia were removed and embedded in moist substrate in separately labelled cups. After adults emerged, they were fed sugar water for 5–10 days to allow the development of wing colour and genitalia. All flies were killed (either on dry ice or in 95% ethanol) and stored at –80 °C in 95% ethanol.

Field observations

To find out if flies court and mate on host plants, we chose a single plant ('plant #71') of *G. spinulosa* bearing 46 accessible male inflorescences. At least three people observed the plant continuously for nine days (9–17 March 2005) from 08:00 to 18:00 h (i.e. ≥ 270 'person-hours' of observation). We used two SONY digital 8 DCR-TRV 280 camcorders to videotape courtship behaviour. We attempted to capture all copulating pairs of flies, and on the final day of observation we captured all *Blepharoneura* observed on plant #71.

Samples for molecular and phylogenetic analyses

To uncover the diversity and patterns of host-tissue specificity of species infesting *G. spinulosa*, we sequenced 60 flies reared from female flowers, 61 flies reared from male flowers, 14 flies caught on plant #71 (the male *G. spinulosa* under observation), and seven flies reared from fruit of *G. spinulosa*. Because the flies that feed on seeds turned out to be morphologically and molecularly very different from the flower feeders, the seed feeder was used as one of the outgroup taxa. Female flowers yielding flies included in our sample were collected from branches found in eight clusters (each cluster probably represents a single plant; Fig. 1): six clusters of female branches were found in the disturbed area near the botanical garden (38 flies), one cluster was found between the botanical garden and JSBS (eight flies), and two clusters were found east of the biological station (14 flies). Male flowers yielding flies included in our sample were collected from 23 clusters (Fig. 1): 14 clusters of male flowers were found in the disturbed area near the botanical garden (42 flies, including 15 flies reared from plant #71), one cluster was found 7.7-km west of the JSBS (two flies), five clusters were found within 1 km of JSBS (11 flies), and four clusters were found more than 1-km east of JSBS (six flies). In addition, we included two species of *Blepharoneura* as the outgroup for all phylogenetic analyses: the sympatric seed-feeding specialist, reared from *G. spinulosa*, and a more distantly related allopatric species, reared from stems of *Sechium pittieri* (Cogn.) C. Jeffrey from San Gerardo de Dota, Costa Rica, which is a member of the *femoralis* group of *Blepharoneura* (Norrbom & Condon, 1999).

Molecular and phylogenetic analysis

Extraction and sequencing

To extract DNA, two legs from each fly were ground in a 1.5-mL microcentrifuge tube using mini pestles (USA Scientific) in 180 μL of PBS buffer (50 mM KPO_4, 150 mM NaCl, pH 8). The DNeasy kit (Qiagen) was then used to extract DNA from the ground tissue. Extracted DNA was stored at –80 °C. The mitochondrial COI was amplified using the primers TL2-N-3014R (Simon *et al.*, 1994) and TY-J-1460 (5′-TACAATCTATCGCCTAAACTTCAGCC-3′), and a Gradient Mastercycler thermocycler (Eppendorf Scientific, Inc.) with the following 'touch-down' program: initial denaturation for 2 min at 92 °C, 12 'touch-down' cycles from 58 to 46 °C (10 s at 92 °C, 10 s at 58–46 °C, 1.5 min at 72 °C), 27 cycles of 10 s at 92 °C, 10 s at 45 °C, and 1.5 min at 7 °C, and a final extension for 10 min at 72 °C. PCR products were held at 4 °C overnight and purified using the QIAquick PCR Purification kit (Qiagen).

To sequence the 3′ end of the COI gene (624 bp), primers TL2-N-3014R and C1-J-2183F (5′-CAACA TTTATTTTGATTTTTTGG-3′) were used. Sequencing was carried out using an ABI 3100 automated DNA sequencer and the ABI Big Dye Terminator sequencing kit (Perkin Elmer Applied Biosystems), and by Macrogen, Inc. (Seoul). Contigs were assembled and aligned with Sequencher (Gene Codes Corp.). Alignment of COI sequences was accomplished by eye, and no indels were required to achieve the alignment. Genetic diversity levels were determined by calculating absolute and corrected *P* distances in PAUP* 4.0 (Swofford, 2002).

Haplotype and phylogenetic analyses

To investigate patterns of variation among haplotypes of *Blepharoneura* infesting flowers of *G. spinulosa*, we used TCS version 1.21:3 (Clement, Posada, & Crandall, 2000) to estimate a haplotype network, using 624 bp of the mitochondrial COI dataset for the 135 specimens that constitute the ingroup for phylogenetic analysis. The connection limit of the TCS analysis was set to 59 steps.

For phylogenetic analyses, we used a pruned dataset containing only a single representative of each haplotype found during this study. This dataset was analysed using maximum parsimony (MP) and maximum likelihood (ML), as implemented by PAUP*4.0b10 (Swofford, 2002). The MP analysis was conducted using a branch and bound search. The dataset was bootstrapped under the MP criterion using branch and bound searches of 1000 pseudoreplicated datasets. For ML analysis, MODELTEST version 3.7 (Posada & Crandall, 1998) was used to determine the model of nucleotide substitution that fitted the data best. The hierarchical likelihood ratio test (hLRT) implemented in MODELTEST selected the GTR+I model (rates = equal, proportion of invariable sites = 0.6384) as the best fit for our dataset. The ML analysis was conducted using a heuristic search with 1000 random sequence addition replicates. The data set was bootstrapped under the likelihood criterion using a fast stepwise-addition search of 100 pseudoreplicates. Posterior clade probabilities were calculated using MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). For this analysis, MrModeltest version 2.2 (Naylander, 2004) was used to select a model of nucleotide substitution compatible with MrBayes. The AIC test, as implemented through MrModeltest, selected the GTR+I model (rates = gamma; proportion of invariable sites = 0.6339) as the best fit for our dataset. The dataset was analysed using 2 000 000 Markov chain Monte Carlo (MCMC) replications with a burn-in of 25%.

Morphological analysis

Using sequenced specimens, we evaluated the wing characters and male genitalic characters that had proved most useful in identifying species of *Blepharoneura* reared from *G. spinulosa* from northern Venezuela (Condon & Norrbom, 1994). For morphological work, we prepared one wing of each sequenced specimen with fully expanded wings. Wings were mounted in euparol on glass slides and were photographed. To analyse wings, we identified a set of over 20 pigmentation-pattern characters. Each individual was then scored for these characters as present or absent, and from these a matrix of frequencies of the most useful characters was assembled for all clades.

To compare clades on the basis of wing patterns, we used a correspondence analysis to determine whether the frequencies of elements of wing pigmentation differed among the clades (Legendre & Legendre, 1998). The results of correspondence analysis can be visualized as an ordination, allowing similarities and differences among clades to be more readily identified. Clades that are most similar in their wing spot frequencies are in close proximity, and clades that are most different in the frequencies of their traits are farther apart. An associated χ^2 test was used to determine whether the distribution of wing spot frequencies differed among clades (see Legendre & Legendre, 1998).

Because epandria were also useful in distinguishing species reared from *G. spinulosa* in Venezuela (Condon & Norrbom, 1994), we used scanning electron microscopy to examine the epandrium from one male specimen from each clade revealed through molecular analyses. Specimens were dissected and

Table 2. Absolute genetic distance (above diagonal) and uncorrected *P* genetic distances (below diagonal) between the most common haplotypes of each clade for 624 bp of the mitochondrial cytochrome oxidase I (COI) gene

Haplotype	A1	B1	C1	D1	E1	F1	Seed
Group	*A*	*B*	*C*	*D*	*E*	*F*	*seed*
A1	*0.002–0.010*	46	49	46	46	52	73
A	(*0.0048*)						
B1	0.073	*0.002–0.003*	52	49	50	57	83
B		(*0.0024*)					
C1	0.079	0.083	*0.002–0.008*	53	53	63	78
C			(*0.0075*)				
D1	0.074	0.079	0.085	*0.002–0.012*	45	57	81
D				(*0.0088*)			
E1	0.074	0.080	0.085	0.072	0.002	61	84
E							
F1	0.083	0.091	0.101	0.091	0.098	*0.002–0.003*	80
F						(*0.0021*)	
Seed	0.117	0.133	0.125	0.130	0.135	0.123	*0.003*
seed							

A1–F1 correspond to the haplotypes shown in Fig. 2. 'Seed' represents the seed-specializing *Blepharoneura* that are the closest outgroup to the flower-feeding flies. *A*-*F* and *seed* refers to haplotype groups: numbers in italics along the diagonal represent the range and the mean (in parentheses) of uncorrected *P* genetic distances between haplotypes (e.g. A1–A2) within each haplotype group (e.g. *A*, *B*, *C*).

epandria were critical-point dried, mounted on stubs, and sputter coated. Digital images were captured on a JEOL 5800LV scanning electron microscope at the Bessey Microscopy Facility at Iowa State University. Although female terminalia were not useful in distinguishing sympatric species in Venezuela (Condon & Norrbom, 1994), we examined female terminalia because they are often used to diagnose tephritid species. Using the same techniques we used for male terminalia, we prepared the eversible membrane and aculeus of a single female from each of two clades. The female terminalia of a specimen from a third clade was not sputter coated, but was examined by SEM under low-vacuum conditions.

RESULTS

Collection and rearing

In 1998 we reared 122 flies from flowers of *G. spinulosa*: 24 flies from female flowers and 98 flies from male flowers. In the expanded study in March 2005, we collected 4704 flowers and 330 fruit from eight cucurbit species in three genera (Table 1). *Blepharoneura* larvae emerged from the reproductive organs of only two species (Table 1): *G. spinulosa* ($N = 767$ larvae) and *Gurania eriantha* (Poepp. & Endl.) Cogn. ($N = 12$ larvae).

Flies reared from *G. eriantha* flowers are morphologically distinct from flies reared from either fruits or flowers of *G. spinulosa*, and will not be considered further. Flies reared from the seeds of *G. spinulosa* also differ morphologically from the flies infesting the flowers of *G. spinulosa*, and are used in the present study as one of the two outgroup taxa. Their outgroup position relative to the flower feeders is corroborated by genitalic characters (see Morphology, below), and by the phylogenetic analysis of sequence data from both mitochondrial and nuclear genes (M. A. Condon, S. J. Scheffer, M. L. Lewis & S. M. Swensen, unpubl. data).

The ingroup of the present study comprises flies reared from either male or female flowers of *G. spinulosa*. We sequenced a 624-bp fragment of the 3′ end of the mitochondrial COI gene from 135 specimens reared from male ($N = 61$) or female ($N = 60$) flowers of *G. spinulosa*, or caught on male plant #71 ($N = 14$). We found 23 different haplotypes represented in this group, with uncorrected pairwise distances ranging from 7.2 to 10.1% (Table 2).

Sequences have been deposited in GenBank. The ingroup taxa are represented by accession numbers EF531754–EF531888, and the two outgroup taxa are represented by EF531751 (*femoralis* group) and two haplotypes of the seed feeder – EF531753 and EF531889–EF531894.

Haplotype and phylogenetic analysis

The haplotype network analysis of 624 bp of the mitochondrial COI dataset (Fig. 2) revealed six distinct groups (hereafter referred to as clades A–F), which correspond to the clades revealed through phyloge-

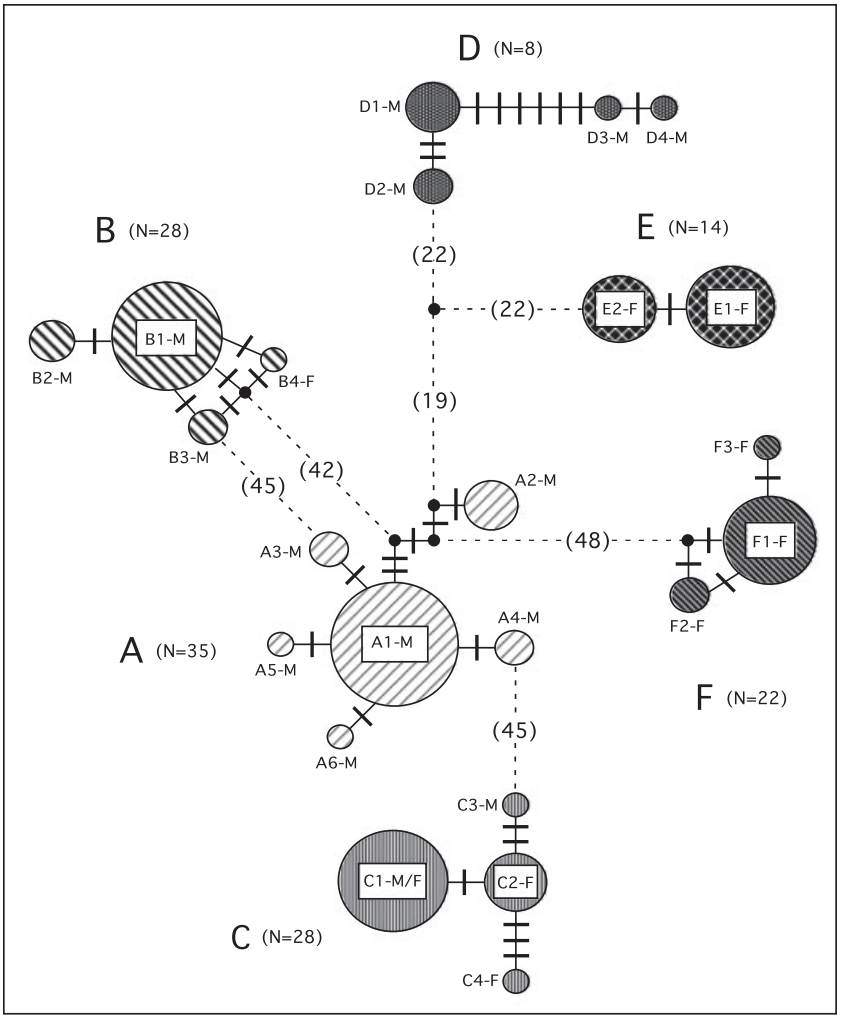

Figure 2. Haplotype network estimated with TCS version 1.21:3 (Clement *et al*., 2000) using a 59-step connection limit. Different haplotype groups are assigned different letters; the numbers following letters are inversely related to the relative abundance of haplotypes within groups (e.g. A1 is the most common haplotype). The circle size is proportional to the sample size; M, flies reared from male flowers; F, flies reared from female flowers; M/F, flies commonly reared from both male and female flowers. Tick marks indicate single base-pair substitutions between closely related haplotypes; dashed lines with numbers in parentheses indicate the numbers of nucleotide substitutions between more divergent haplotype pairs.

netic analyses (Fig. 3). These haplotype groups were internally quite homogeneous (average uncorrected pairwise distance within groups = 0.46%), but also differed considerably from each other (uncorrected pairwise distances among groups range from 7.2 to 10.1%) (Table 2). The number of haplotypes per group ranged from two (clade E) to six (clade A); three clades included four haplotypes (clades B, C, and D). Clade A ($N = 35$) included six haplotypes, the most divergent of which differed by 5 bp from the most common haplotype: four of the six clade-A haplotypes come from seven flies captured on a single host plant (plant #71; Table 3). Clade D, represented by the fewest individuals, included the most divergent haplotypes; half of the individuals in clade D (including the most common and the most divergent haplotypes) were collected from a single host plant (plant #71; Table 3).

The phylogenetic analysis of the dataset containing one representative from each haplotype produced eight trees using MP (Fig. 3A) and a single tree using ML (Fig. 3B). Both MP and ML trees revealed the same six clades, but differed in the relationships among these clades. The six clades correspond to haplotype groups A–F identified by the haplotype network analysis (Fig. 2), and are supported by high bootstrap and posterior clade probability values; however, branches that differ between MP and ML analyses have either weak or no support. The eight

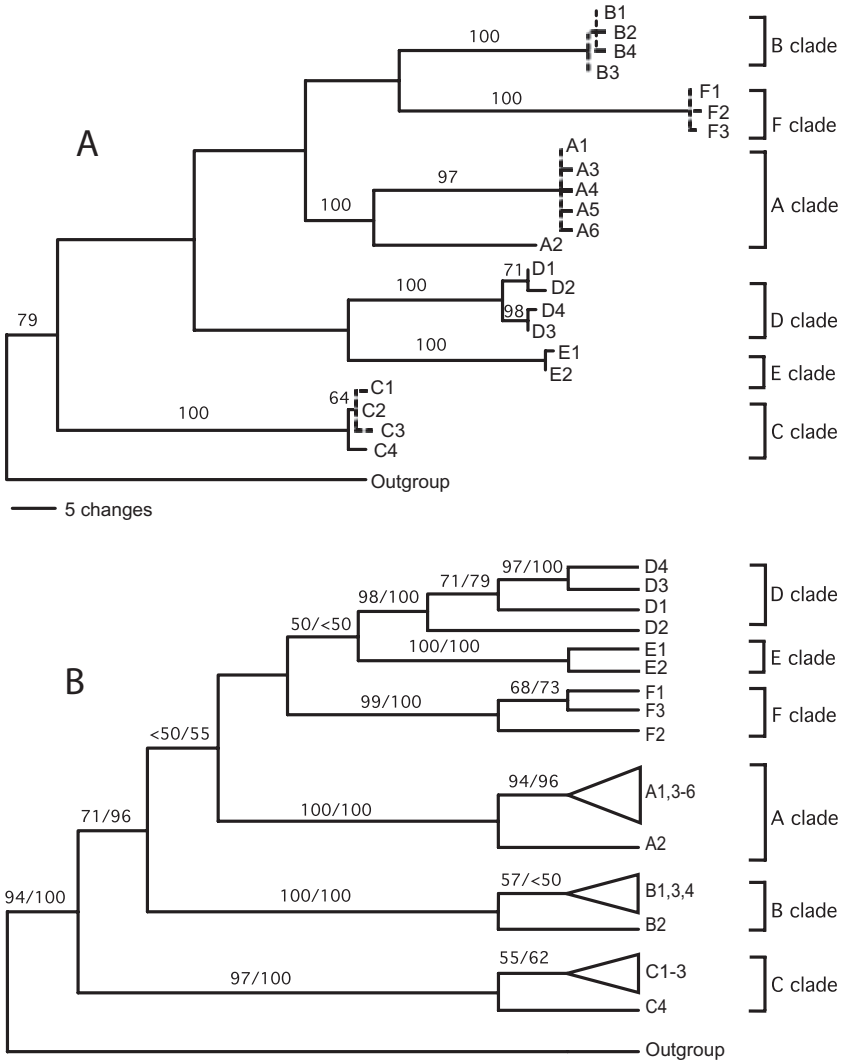

Figure 3. Phylogenetic analyses of 624 bases of the cytochrome oxidase I (COI) mitochondrial gene. Trees generated using maximum parsimony (MP) and maximum likelihood (ML) reveal the same six clades, but differ in the relationships among clades. A, one of eight optimal MP trees (tree length, TL = 300, excluding autapomorphies; consistency index, CI = 0.66; retention index, RI = 0.86; rescaled index, RC = 0.58). Dashed lines indicate branches that differed among the eight trees. Bootstrap values are shown above the branches. B, single ML tree (–lnL = 2185.50853) with likelihood bootstrap values (before the slash mark) and Bayesian posterior clade probabilities (after the slash mark) shown for each branch. Each triangle represents a clade with no resolution among its members. For both trees, branch support values below 50% were not reported.

Table 3. Flies (haplotypes) caught or reared from plant #71 (male *Gurania spinulosa*)

Clade	Copulating individuals	Solitary individuals	Reared from male flowers
A	4: one pair (♀A1, ♂A4) on male inflorescence, one pair (♀A1, ♂A4) on a leaf	3: 2♂ (A5, A2); 1♀ A1 on inflorescence	0
B	2 (B1) on leaf	3: 2♀ B1 on same leaf, 1♀ B3 on inflorescence)	10 (9B1, 1B3)
C	0	0	3 (C1)
D	2 (♀D1, ♂D4) on leaf	0	2 (D1)
E	0	0	0
F	0	0	0

Table 4. Number of individuals reared from male or female flowers of *Gurania spinulosa*

Clade	Male flowers (# plants)	Female flowers (# plants)
A	26 (16)	2 (2)
B	22 (11)	1 (1)
C	7 (5)	21 (4)
D	6 (5)	0
E	0	14 (4)
F	0	22 (5)

The numbers of clusters of flowers (~ number of plants) are given in parentheses. Clades differ significantly in their tendencies to infest male versus female flowers ($\chi^2 = 88.743$, d.f. = 5, $P < 0.0001$).

MP trees differed only in their placement of closely related taxa within clades F, C, and A. The ML tree provided no resolution among closely related taxa in clades A, B, and C (Fig. 3).

Patterns of host use

Flies that form the six ingroup clades are both host plant taxon specialists and host-part specialists on the flowers of *G. spinulosa*: none was reared from other species of cucurbits or from fruit, despite extensive sampling of the available cucurbits (Table 1). Three of the six clades of *G. spinulosa* flower specialists represent specialists on male flowers (Table 4): clade A (92.9%; $N = 26$ of 28 reared flies); clade B (95.7%; $N = 22$ of 23); clade D (100%; $N = 6$). Two of the six clades represent specialists on female flowers: clade E ($N = 14$) and clade F ($N = 22$) include flies reared only from female flowers. In clade C, 75% of specimens ($N = 21$ of 28) were reared from female flowers; the remaining 25% were reared from male flowers.

As many as three clades were found infesting the flowers of a single individual host plant. Multiple individuals of three clades (B, C, and D) were reared from male plant #71 (Table 3). Of the five clusters of female branches from which we sampled at least three flies, three yielded three clades (C, E, and F) and two yielded two clades (C and F). Although multiple clades often infest different flowers of a single plant, multiple infestations of single flowers are rare. Of the 624 male flowers of *G. spinulosa* that yielded puparia, only four yielded two puparia: one of those (from plant #71) yielded two clades (B and D).

Observations of behaviour

Four pairs of flies were captured *in copulo* on the single male *G. spinulosa* plant #71, and those eight flies represent three distinct clades of specialists on male flowers (Table 3). In all cases, both members of the copulating pairs belong to the same clade. Two copulating pairs belong to clade A: one of those pairs copulated on a leaf; the other pair courted and copulated on a male inflorescence, where the male displayed a behaviour we call 'clap' (Condon & Norrbom, 1999). Clap displays include extremely rapid wing motions that are apparent as a blur in frozen 1/30-s video frames, but are not visible (even as a blur) in real time. The male of the third copulating pair (clade B) also displayed a 'clap' but on the surface of a leaf (not on an inflorescence), where copulation also took place. During the clap display, wings are not outstretched but are held in an orientation similar to the position of the wings of a fly at rest (Fig. 4A).

The fourth pair (clade D) copulated on a leaf after the male displayed a behaviour we call 'shimmy', a rapid version of the display called asynchronous supination that is commonly displayed by tephritids (Headrick & Goeden, 1994). In this semaphore-like display, flies alternately rotate and outstretch each wing (Fig. 4b). Wings are rotated so that the costa (anterior wing margin) is perpendicular to the long axis of the body, and the ventral plane of the wing faces forward and is perpendicular to the substrate. More detailed and quantitative descriptions of displays will be reported elsewhere (J. Gammons & M. Condon, unpubl. data).

In addition to courting, adults of *Blepharoneura* spend a considerable period of time abrading and feeding on the surfaces of young leaves of *G. spinulosa*. Adults were also observed on inflorescences, where females oviposit into calyces of flowers. Males appear to 'patrol' male inflorescences (where copulation by members of at least one clade, clade A, was observed; Table 3). On the final day of observations, six individuals were captured on plant #71 (Table 3): four individuals were captured on male inflorescences (two males and one female of clade A, and one female of clade B), and two females of clade B were captured at different times while feeding on the same young leaf. Although more flies of clade A ($N = 7$) were captured as adults than any other clade on plant #71, no flies of clade A emerged from flowers of that individual plant (Table 3).

Morphology

Wing pigmentation pattern

After screening more than 20 wing pigmentation characters on 133 wings from flies in all six clades, we found no fixed elements of pattern that could be used as diagnostic characters (Figs 5, 6; Table 5). For example, the character 'spots 26 and 27 *not* fused' was fixed in three flower-feeding clades (A, B, and D), was

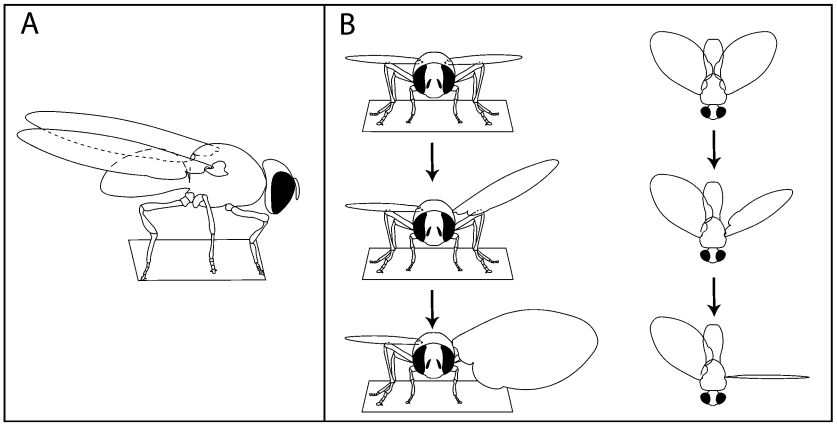

Figure 4. Wing positions during behaviours. A, clap (sideview): wing movements during the clap behaviour occur when the wings are held back over the abdomen; anterior wing margins are initially held away from the midline; during a 'clap' the anterior margins move very rapidly towards the midline. B, shimmy (front and top views): the motions shown for one wing are repeated alternately and rapidly with the other wing; wings are rotated forward, with the ventral side facing forward.

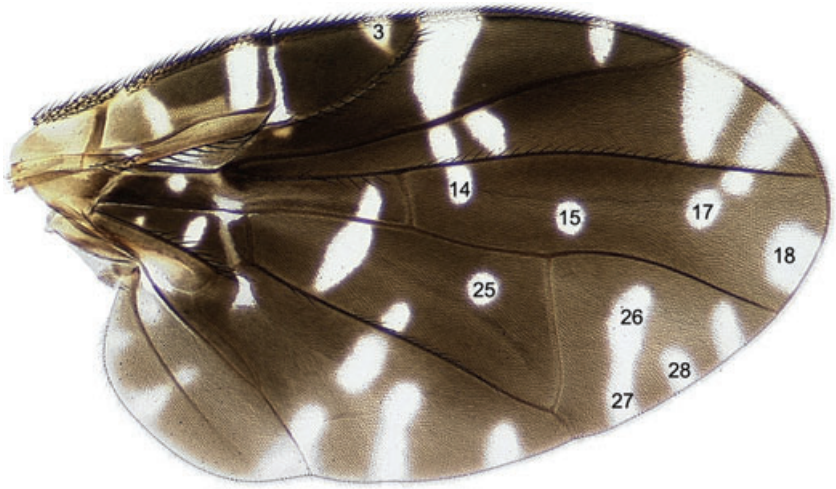

Figure 5. Wing of a fly reared from seeds of *Gurania spinulosa*. The wing spots that are useful for distinguishing species that feed on flowers of *G. spinulosa* are labelled with numbers (Condon & Norrbom, 1994).

absent from seed feeders, and was variable in three clades (C, E, and F). In contrast, 'spot 25 ≥ spot 15' was fixed in clades C, E, and F, and was variable in clades A, B, and D (Table 5). Although no single element was useful as a diagnostic character, clades differed significantly in the frequencies of different elements of wing pigmentation pattern (Figs 6, 7; Table 6).

Correspondence analysis revealed a significant difference among clades with respect to the frequency of wing pattern elements ($\chi^2 = 213.272$, d.f. = 25, $P < 0.0001$). Most clades (all but clade D) differed significantly from one another (Table 6). For instance, clades F and C were conspicuously distinct from the remaining clades (Fig. 7): clade F had higher frequencies of spots 26–27 fused and spot 18-touch-M, and lower frequencies of spot 15 < spot 14, relative to the other clades (Table 5). Although clade D (represented by only eight specimens) differed significantly from three clades (C, E, and F), and differed marginally from clade A ($P = 0.0038$), it did not differ significantly from clade B (Table 6). The correspondence analysis ordination plot explained 84% of the variance in wing pattern, and graphically displays relationships among frequencies: clades closer together (e.g. B and D) are most similar; clades farthest apart differ most. From this analysis it is evident that the relative frequencies of different wing pigmentation traits differ among

Table 5. Frequencies of wing pattern elements (Fig. 5) differing among sympatric species in eastern Ecuador (Fig. 6)

Clade (= species)	*N*	Spot 18 touch M: not	Spot 28 +:0	Spot 17 +:0	Spot 25 < spot 15: 25 ≥ 15	Spot 15 < spot 14: 15 ≥ 14	Spots 26–27 fused: not
A	35	1:34	29:6	**18:17**	**11:24**	30:5	0:35
B	27	0:27	21:6	0:27	**17:10**	27:0	0:27
C	27	0:27	**1:26**	**12:15**	0:27	23:4	**10:17**
D	8	0:8	**1:7**	0:8	**7:1**	8:0	0:7
E	14	0:14	14:0	1:13	0:14	**7:7**	3:11
F	22	**11:11**	20:2	0:22	0:22	**3:19**	**19:3**
Seed	7	0:7	5:2	**6:1**	0:7	**2:5**	**7:0**

Characters that are most distinctive for particular clades are set in bold. Variables are counted as follows: touching vein M or not; present (+) or absent (0); size relative to another spot (<, ≥); spots fused or not. 'Seed' represents the seed-specializing *Blepharoneura* used as the outgroup in the phylogenetic analysis (Fig. 5).

Table 6. χ^2 values (below diagonal) and significance levels (above diagonal) associated with pairwise comparisons of frequencies of wing pigmentation characters (Figs 4, 5; Table 5)

	A	B	C	D	E	F
A	–	0.0010	0.0001	**0.0038 NS**	0.0012	0.0001
B	18.431	–	0.0001	**0.0924 NS**	0.0004	0.0001
C	43.922	58.263	–	0.0001	0.0001	0.0001
D	15.497	4.764	29.677	–	0.0003	0.0001
E	20.167	20.421	58.263	21.388	–	0.0009
F	75.824	65.683	57.048	51.403	18.647	–

Significance level set as $P < 0.00333$ through Bonferroni correction. NS, not significant.

clades, although no single wing spot character completely differentiates these clades.

Terminalia

Examination of male terminalia revealed subtle differences in shape and setae number of the inner surstylus of the epandria (Fig. 8). For example, the prensisetae of clade-A males are closer together than the prensisetae of males in most other clades, and the inner surtylus bears more setae. In clade C, the distance between the prensisetae is intermediate between clade A and the other clades, and bears only two setae. The outer prensisetae of clade B is blunter than those of the other three clades (D, E, and F) with widely spaced prensisetae. Two of those clades (F and E) are difficult to distinguish: both have pointed outer surstyli and numerous setae. The third clade with widely spaced prensisetae (clade D) has a longer pointed outer prensiseta. These characters are difficult to assess quantitatively under a light microscope, and may not prove useful in distinguishing clades. Without examining more specimens under SEM, we are not confident that these characters represent fixed differences. In contrast, the epandrium of the seed-feeding flies (Fig. 9) has a third lobe on the inner surstylus (like *Blepharoneura manchesteri* Condon & Norrbom) and clearly differs from that of the flies reared from flowers.

Preliminary examination of female terminalia of three clades (A, C, and D) prepared for SEM revealed trivial differences in the length and width of the tip of the aculeus, and essentially no difference in the number of teeth along the margin of the aculeus (clades A and C, 37 teeth; clade D, 36 teeth). The aculeus tip resembles the tips of species reared from flowers of *G. spinulosa* in northern Venezuela (Condon & Norrbom, 1994).

DISCUSSION

Extraordinary diversity

This study of *Blepharoneura* reveals a surprising level of diversity in flies that feed as larvae specifically on flowers of a single species of host in a small area in eastern Ecuador (Fig. 1). Our reared samples of flies from the flowers of *G. spinulosa* harboured extensive mitochondrial variation, exhibiting a high degree of phylogenetic structure. The six clades recov-

Figure 6. Wings of four individuals for each of the six flower-feeding species. Boxes drawn on wings indicate patterns typical of particular species (see Fig. 7; Table 5). Wings were chosen to show intraspecific variation in elements of pigmentation pattern. A, spot 17, variable; relative sizes of spots 25 and 15, variable. B, spot 17, absent; spot 25–15, relative size variable. C, spot 17, variable; spots 26–27, variably fused; spot 28, absent. D, spot 17, absent; spot 25, smaller than spot 15; spot 28, absent. E, spot 17, absent; spots 26–27, not fused; spot 28, present. F, spot 18, touches vein M, variable; spots 26–27, fused; spot 28, present; spot 15, at least as big as spot 14.

ered in the haplotype and phylogenetic analyses are strongly supported by the available molecular data. Intraclade variation is low, whereas variation among clades is much higher (Figs 2, 3, Table 2), a pattern strongly indicative of the presence of distinct species (e.g. Scheffer & Wiegmann, 2000; Hebert *et al*., 2004). However, to conclude that these six clades unequivocally represent different species, we must have corroborating evidence from an additional data source, such as morphology, behaviour, host use, or an unlinked locus. Although the importance of corroborating evidence is sometimes overlooked in attempts to use mitochondrial sequence data to delimit species, it remains critical to a thorough examination of organismal diversity.

Our initial explorations of other data sources are consistent with the hypothesis that the six clades recovered during this study represent distinct species. The six clades occupy significantly different morphological spaces, defined by wing pigmentation patterns (Fig. 7, Tables 5, 6); however, no single diagnostic wing pigmentation character was discovered. Males belonging to different clades were observed engaging in different courtship behaviours, and the members of each of the four pairs of flies captured *in copulo* always belonged to the same clade. These observa-

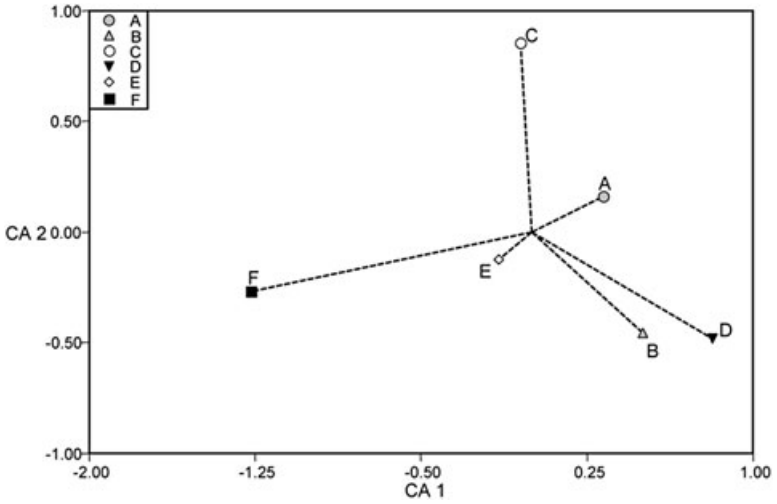

Figure 7. Ordination from a correspondence analysis of the frequencies of elements of wing pigmentation for the six haplotype groups (Figs 5, 6; Table 5). The first two ordination axes explain 84% of the total variation. Haplotype groups are designated their tentative labels, A–F. Haplotype groups that are dissimilar in the frequencies of elements of wing pigmentation are found in different directions (e.g. F, C, and A), whereas haplotype groups (e.g. B and D) with similar patterns in frequencies are plotted close together.

tions suggest assortative mating by clade, even in the presence of flies of multiple clades on the same individual host plant (plant #71). Finally, preliminary data from two nuclear genes (M. A. Condon, S. J. Scheffer, M. L. Lewis & S. M. Swensen, unpubl. data) are consistent with the hypothesis of six species of *Blepharoneura* infesting the flowers of *G. spinulosa* in the Jatun Sacha region of Ecuador. Because all of our data sources consistently support this hypothesis, in the remainder of this article we will use the terms 'species' and 'clades' interchangeably.

Our confidence in the status of these clades as distinct species is underscored by their close coexistence in sympatry. Paradoxically, the very limited geographical range of the sample reported here, which clearly shows that these are distinct species, is precisely the reason we choose not to assign formal names to these species. Several of the species we discovered (A, C, and F) are members of geographically widespread monophyletic mitochondrial DNA (mtDNA) COI lineages, comprising multiple geographically restricted monophyletic groups (M. A. Condon, S. J. Scheffer, M. L. Lewis & S. M. Swensen, unpubl. data). Using some phylogenetic species concepts (Baum & Donoghue, 1995; Baum & Shaw, 1995) we could treat each of those smallest monophyletic groups as species, but the discovery of those groups requires samples from sites throughout the distribution of *Blepharoneura*. Samples from throughout the Neotropics are necessary to determine whether allopatric populations exist, and if they exist, whether they form reciprocally monophyletic groups that could be called species, or represent widespread species that do not form distinct allopatric monophyletic groups. For this reason, we defer formal naming of new species until we have a more complete geographical sample.

Patterns of host use

The six sympatric species of *Blepharoneura* that are the focus of this study are all highly host specific (at least in this location): all individuals were reared exclusively from *G. spinulosa*, despite extensive sampling from other potential hosts (Table 1). These species are not only specific to a single host taxon, they are also specific to particular host tissue – larvae feed only on the flowers. Of these six species, five are specialists on specific flower sexes (Table 4): two species feed exclusively on female flowers; three species feed as larvae mainly on male flowers. The sixth species (C) is a generalist on both male and female flowers. All species feed primarily on calyx tissue and do not appear to specialize on gender-specific tissues: larvae in female flowers rarely enter ovaries or cause abortion of flowers; larvae in male flowers complete their development after anthesis and abscission of the flowers. Thus, flower feeders have no obvious impact on the fitness of the host.

Behaviour and morphology

Courtship and mating of at least three of the flower-feeding *Blepharoneura* species (A, B, and D) occur on the surface of the host plant. Courtship displays, including the exact location of the displays on the

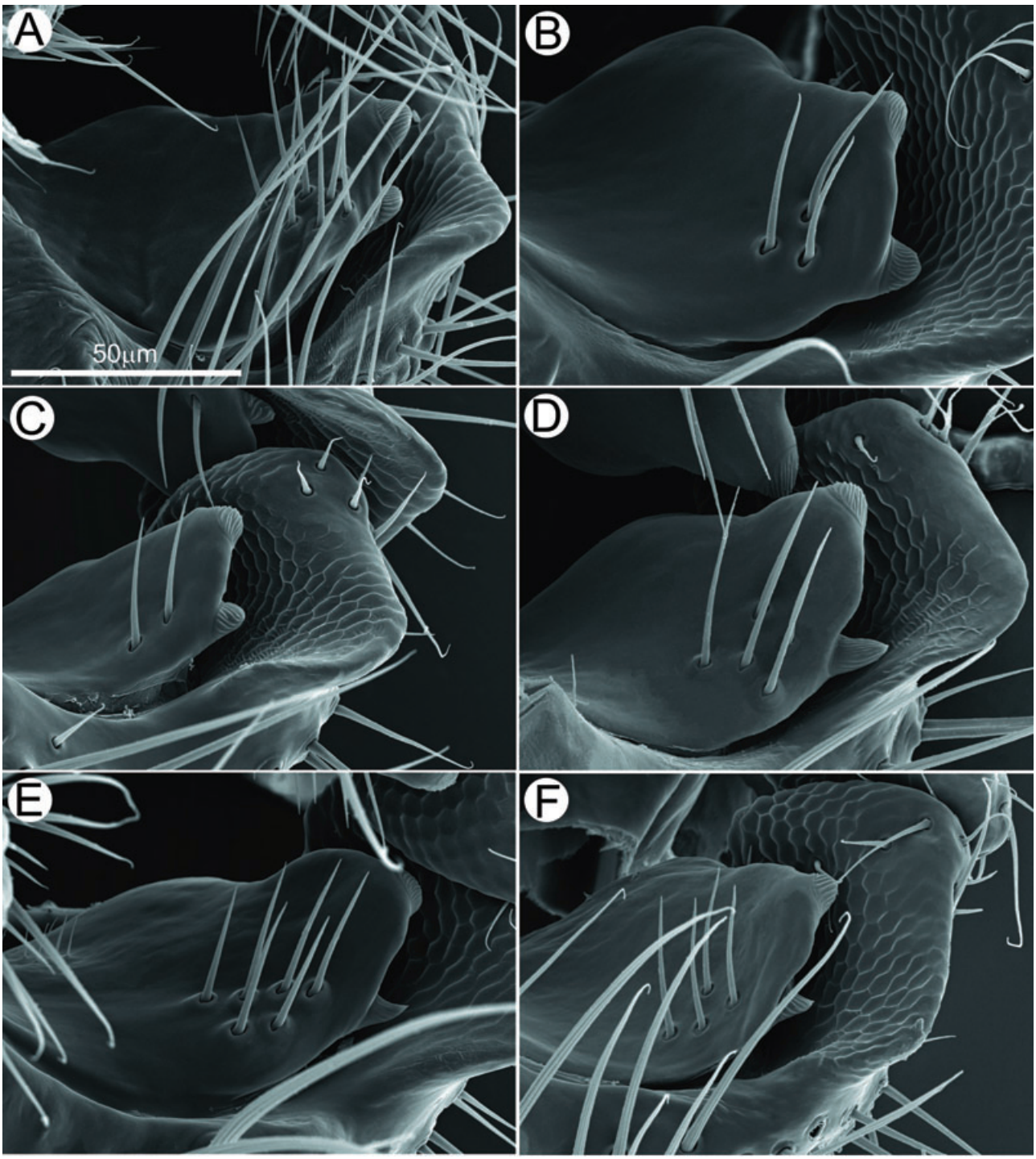

Figure 8. Scanning electron micrographs (SEM) of the inner surstyli of the epandrium of each of the six flower-feeding species. Letters refer to clades revealed through phylogenetic analyses (Fig. 3). A, both prensisetae are blunt and close together; numerous setae on inner surstylus. B, blunt prensisetae, distant from each other; three setae. C, blunt prensisetae, somewhat close together. D–F, lateral prensiseta pointed, and prensisetae far apart.

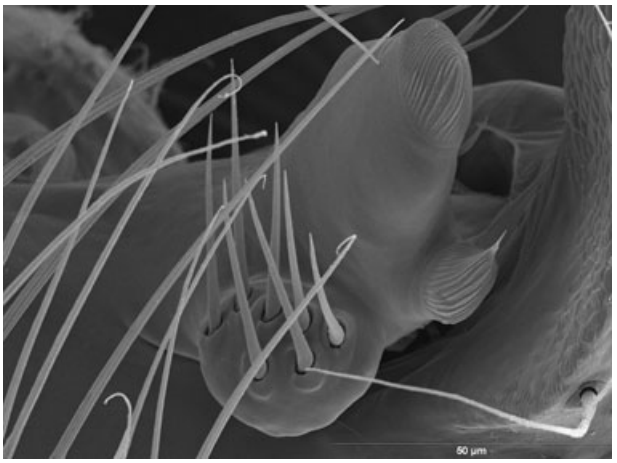

Figure 9. Scanning electron micrograph (SEM) image of the inner surstylus of the epandrium of the seed-feeding fly, with distinctive lateral lobe bearing numerous setae.

host plant, behavioural features, and morphological characteristics, may play key roles in maintaining reproduction isolation among sympatric species of *Blepharoneura* using the same host species.

Wing movements play an important role in the courtship displays of many flies (Sivinski *et al*., 1999; Wilkinson & Johns, 2005). We observed two distinctive wing motions in displays of these flies observed in the field: 'clap' and 'shimmy' (Fig. 4). The clap display includes extremely rapid wing motion (Condon & Norrbom, 1999): a frame rate of 1000 frames s^{-1}, with 1/4000 s shutter speed, is required to freeze the wing during a clap display (M. Condon, unpubl. data). Such rapid wing motions occurring when an insect is perched on a plant surface are likely to cause characteristic vibrations, either airborne or substrate-bound (Sattman & Cocroft, 2003). Acoustic or vibrational communication is common among insects, including other tephritids (Burk, 1981; Sivinski & Webb, 1985; Ewing, 1989; Sivinski *et al*., 1999; Alonso-Pimentel *et al*., 2000), and may facilitate divergence following host shifts (Rodriguez *et al*., 2004): transmission of vibrations through plants can be affected by differences among host plant species, as well as by differences among different plant parts of a single species (Cocroft & Rodriguez, 2005; Cocroft *et al*., 2006).

Wing movements during courtship may also involve visual cues that could affect the evolution of pigmentation patterns (Singh & Chatterjee, 1987; Faust & Brown, 1998; Kopp & True, 2002). In some species of *Blepharoneura* (e.g. clade D; Fig. 4b), males display with outstretched wings in front of females (Condon & Norrbom, 1999). If females respond to the pigmentation pattern visible during such displays, evolution may occur rapidly and lead to the fixation of those pattern elements. In northern Venezuela, wing pigmentation patterns include fixed (or nearly fixed) elements that are useful as diagnostic field characters for all three *G. spinulosa* feeders found there (Condon & Norrbom, 1994). In contrast, flies reared from flowers of *G. spinulosa* in eastern Ecuador are exceedingly difficult to distinguish morphologically using wing pigmentation characters (Figs 4–6; Tables 5, 6).

In species that perform rapid wing displays while poised behind females (e.g. the 'clap' display in *Blepharoneura*), vibrational cues may be more important than visual cues. Such differences in displays (and in the responses of females to the displays) may help account for differences in the relative importance of pigmentation patterns in different species of *Blepharoneura*, just as in *Drosophila* (Kopp & True, 2002). Selection on species-specific courtship displays may also account for subtle, but significant, differences in wing shape among these same six sympatric species of *Blepharoneura* found in eastern Ecuador (S. Marsteller, D. C. Adams, M. L. Collyer & M. A. Condon, unpubl. data).

Implications for diversification via host shifts

Could local shifts in host-part use explain the evolution of the lineages we have uncovered? Enemy-free space can favour host shifts in diverse insects (Lill, Marquis, & Ricklefs, 2002; Oppenheim & Gould, 2002; Murphy, 2004), including other tephritids (Brown *et al*., 1995). In our sample, mortality caused by parasitoids differs dramatically between flies emerging from male versus female flowers: parasitoids emerged from 16.4% of puparia from mature male flowers ($N = 238$ puparia yielding adult flies or parasitoids), and from 29.5% of the puparia from mature female flowers ($N = 44$) (J. Johnson & M. Condon, unpubl. data). Thus, parasitoids may represent a selective pressure that could favour shifts from female to male flowers.

Competition for resources can also favour ecological diversification (Denno, McClure, & Ott, 1995; Schluter, 2000); however, we found no evidence for resource limitation: many flowers contain no larvae (Table 1), and flowers infested by more than one larva are very rare, but fit the expectations of a Poisson distribution (J. Johnson & M. Condon, unpubl. data). These species of *Blepharoneura* commonly co-occur in different flowers on the same plant individual, and occasionally can even be found within the same flower. An apparent exception to this pattern is species A: seven adults, including two copulating pairs, were caught on plant #71. However, no individuals of species A were reared from flowers of plant #71 (Table 3). We did not find any individuals of species A in the same flower with another species; however, we did rear conspecifics from single flowers. These observations suggest that some

form of interspecific interaction may affect the distribution of these species.

Both interspecific and intraspecific interactions deserve closer attention, especially during other times of year: abundance and sex ratios of flowers of *G. spinulosa* fluctuate seasonally (Condon, 1984). We sampled during the wet season, when flowering of *G. spinulosa* peaks and female flowers are most abundant; resource limitation may occur during the dry season, when flowers are rare and sex ratios are highly male biased. During periods of low resource availability, shifts from female flowers to more abundant male flowers could be advantageous. Although both competitive interactions and predation by parasitoids could favour shifts in host use, divergence resulting from repeated host shifts in sympatry seems unlikely – especially among sympatric populations that court and mate on the same host plants. Instead, we suggest that speciation, perhaps accelerated by sexual selection, may occur in allopatry when the habitat of the widespread host plant is fragmented. Amazonian South America has a history of fragmentation, and the Napo is one of several biogeographical regions with a history of isolation from other Amazonian regions (Hall & Harvey, 2002). If allopatric populations of host-specific insects diverge more rapidly than host plant populations, diversification of host-specific insects could occur without shifts in host use.

Gurania spinulosa is commonly found throughout tropical South America. Throughout its distribution, *G. spinulosa* is infested by species of *Blepharoneura* (M. A. Condon, S. J. Scheffer, M. L. Lewis & S. M. Swensen, unpubl. data). Preliminary analysis of *Blepharoneura* from diverse localities throughout South America suggests that patterns of host use in *Blepharoneura* may actually be more conservative than either morphology or courtship behaviour (M. A. Condon, S. J. Scheffer, M. L. Lewis & S. M. Swensen, unpubl. data). For example, our survey of diverse geographical regions shows that eastern Ecuadorian species A is more closely related (i.e. differs by only ~1.3% bp) to a species in northern Venezuela than to any of the sympatric species in eastern Ecuador (Table 2). Flies in both Venezuelan and Ecuadorian populations are specialists on male flowers of *G. spinulosa*, but differ in courtship behaviour and wing pigmentation pattern (Condon & Norrbom, 1994, 1999; Condon & Steck, 1997). These observations suggest that courtship behaviours and some morphological characters diverge more rapidly than patterns of host use. The conservatism of host use in *Blepharoneura* appears to be an ancient trait: *Blepharoneura* is one of three genera in the pantropical subfamily Blepharoneurinae, which is one of the oldest lineages in the Tephritidae, and all known hosts for the subfamily are plants in the Cucurbitaceae (Norrbom & Condon, 1999). Flower feeding may also be highly conserved: the only known host record for *Baryglossa*, one of the two palaeotropical genera in the Blepharoneurinae, is from a flower of a cucurbit (Munro, 1957). Although rigorous tests of these hypotheses await further studies of the Blepharoneurinae, available comparative data support the idea that frequent host shifts are not drivers of diversification of *Blepharoneura*.

Implications for diversity

The identification of six species of *Blepharoneura* feeding only on the flowers of a single host plant species in one very small geographical region is startling. The discovery is especially startling in light of evidence that additional morphologically similar species of *Blepharoneura* have been reared from the same host plant species (*G. spinulosa*), and from as many as 12 other species in the subtribe Guraniinae in other regions of both Central and South America (e.g. Condon & Norrbom, 1994; M. A. Condon, S. J. Scheffer, M. L. Lewis & S. M. Swensen, unpubl. data). If these other host plant species also harbour extensive communities of sympatric *Blepharoneura*, as seems to be the case (Condon & Steck, 1997), we have an extraordinary radiation with which to explore the roles of host shifts, specialization, behaviour, morphology, and geography in promoting diversification.

Our future work will use multigene phylogenetic analyses, in combination with behavioural and morphological studies, to test hypotheses about diversification and host-use evolution in this species-rich genus. In particular, we will explore hypotheses that the extraordinary diversity of cryptic species in this group may result from several factors (that also apply more generally to other groups of insects): (1) clade antiquity; (2) distribution over large and repeatedly fragmented geographical areas; (3) elaborate courtship displays involving nonvisual cues; (4) courtship restricted to specific locations (e.g. host plants); (5) high levels of host specificity; (6) shifts in use of host taxa or host parts. Although clade age alone may help explain major patterns of diversity (McPeek & Brown, 2007), particularly in the tropics (Brown, 1988; Farrell & Mitter, 1993; Ricklefs & Schluter, 1993; Willig, Kaufman, & Stevens, 2003; Weir & Schluter, 2007), we predict that the discovery of other complexes of cryptic species in the tropics will underscore the importance of sexual selection in host-specific phytophagous insects as an accelerator of diversification (West-Eberhard, 1983; Mitter, Farrell, & Wiegmann, 1988; Wilkinson & Johns, 2005).

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